RESPONSES OF THE RIBBED MUSSEL, *GEUKENSIA DEMISSA*,
TO THE HARMFUL ALGAE *AUREOCOCCUS ANOPHAGEFFERENS*
AND *HETEROSIGMA AKASHIWO*

EVE GALIMANY, JULIE M. ROSE, JENNIFER ALIX, MARK S. DIXON
AND GARY H. WIKFORS

National Marine Fisheries Service, Northeast Fisheries Science Center, NOAA, 212 Rogers Avenue, Milford, CT 06460, USA

Correspondence: Gary H. Wikfors; e-mail: gary.wikfors@noaa.gov

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ABSTRACT

The ribbed mussel (*Geukensia demissa*) is a keystone species in the salt marshes of the North American Atlantic coast. We investigated the clearance rates (CRs) and pathology of ribbed mussels exposed to cultures of two toxic algae, *Aureococcus anophagefferens* and *Heterosigma akashiwo*, for 5 d and contrasted the results with mussels fed the nontoxic alga *Tetraselmis chui*. We also conducted a separate, *in vitro* experiment in which we exposed extracted mussel haemocytes to the three different algae, their respective media and seawater, as a toxicity bioassay. Results show that mussels exposed to either of the toxic algae had significantly decreased CRs during the entire exposure period. Histopathology revealed thinner digestive tubules, gonad reabsorption and a decrease in gill ciliates in response to both toxic algal species. There was a significant increase in mortality of haemocytes exposed *in vitro* to both toxic algae compared with seawater controls. Results suggest that ribbed mussel feeding behaviour and physiology can be impacted in several ways by harmful algae, which could undermine their contribution to pelagic-benthic coupling processes.

INTRODUCTION

*Geukensia demissa* (Dillwyn, 1817), the Atlantic ribbed mussel, is distributed along the North American Atlantic coast from the Gulf of St Lawrence (Canada) to Florida (USA) (Abbott, 1974). This species is found in intertidal habitats, mainly salt marshes, experiencing a wide range of salinities from oceanic values to 20 and lower (Castagna & Chanley, 1973). Although the ribbed mussel is not a commercially harvested species, this mussel has a very important ecological role in pelagic-benthic coupling. These suspension-feeding bivalves transfer particulate matter from the water column (pelagic) to the benthos, depositing organic phosphorus, nitrogen and carbon onto the sediment surface (Verwey, 1952; Kuenzler, 1961; Doering, Oviatt & Kelly, 1986; Dame, Spurrier & Wolaver, 1989). This process is especially important in shallow-water ecosystems, such as salt marshes, where the ratio of benthic surface area to water volume is high (Dame, 1996). Accordingly, Kuenzler (1961) characterized the ribbed mussel as a keystone species in saltmarsh biogeochemical processes and emphasized the importance of this species in the flow of phosphorus in a salt marsh. Subsequently, Jordan & Valiela (1982) described the important role of ribbed mussels in the environmental flow of nitrogen, with this species releasing more ammonia in salt marshes than any other species. Thus, absence of ribbed mussels in a salt marsh or a disturbance to their physiology may have important consequences not only for the species itself but also for the ecosystem.

Toxic microalgal proliferations in aquatic ecosystems, referred to as harmful algal blooms (HABs), appear to be increasing in geographic distribution and intensity (Hallegraeff, 2003), disrupting coastal and marine ecosystems and the organisms living there (Smayda, 1990; Landsberg, 2002). Bivalves that are filter feeders ingest noxious and toxic algae present in the water. Several biological effects of toxic microalgae upon mussel species, chiefly commercially harvested *Mytilus* species, have been described, including decreased clearance rates (CRs) or even cessation of feeding (Lesser & Shumway, 1993; Bricelj, MacQuarrie & Schaffner, 2001). Nevertheless, the effects of HAB species upon ribbed mussels have not been studied and such effects can be species-specific (Landsberg, 2002). Although many studied HAB species tend to be oceanic or coastal in distribution, some do occur in the saltmarsh habitats of ribbed mussels. One is the ‘New England brown tide’ organism *Aureococcus anophagefferens* (Sieburth, Johnson & Hargraves, 1988). This species has produced episodic, ecosystem-disrupting blooms in several northeastern-USA estuaries, including Narragansett Bay, the south-shore bays of New York’s Long Island, New Jersey coastal embayments and Maryland coastal bays (Casper, Gobler & Benmayor, 1996; Wazniak & Gilbert, 2002).
2004; Gobler, Lonsdale & Boyer, 2005). Mortalities and reproductive failure of bivalves, including *Mysitius edulis*, northern bay scallops *Argopecten irradians* and northern quahogs *Mercenaria mercenaria*, have been observed in bloom-impacted waters (Bricelj & Kuenzler, 1989; Smyda & Fofonoff, 1989; Bricelj & Lonsdale, 1997; Bricelj & MacQuarrie, 2007). Inimical effects on bivalves have been attributed to an anaesthetic effect of the extracellular polymeric substance (EPS) produced by *A. anophagefferens* upon gill cilia, resulting in respiratory impairment (Gainey & Shumway, 1994). Another saltmarsh inhabiting HAB species is the ichthyotoxic raphidophyte *Heterosigma akashiwo*. This organism, and related raphidophyte species, cause gill damage in fish, thought to be mediated by a combination of reactive-oxygen species (ROS) and fatty-acid oxidation products (Marshall, Nichols & Hallegaard, 2002; Marshall et al., 2003). *Heterosigma akashiwo* has also been shown to cause pathologies and impairment of cellular immune defence function in bivalves exposed to cultures in the laboratory (Hégaret, Shumway & Wikfors, 2007).

Ribbed mussels feed on several types of particles suspended in the water column, including phytoplankton, bacteria and detritus; mussels may also absorb dissolved organic material (Jordan & Valiela, 1982; Kreeger & Newell, 1996). Kreeger & Newell (2001) observed that the digestive physiology of ribbed mussels responds to shifts in dietary components during the year, suggesting that mussels efficiently balance their nutritional limitations of the experiments (Riisgaard, 2001). The clearance capacity of ribbed mussels, including *A. irradians* scallops, *Argopecten irradians*, *Mercenaria mercenaria* and *Mytilus edulis*, eastern oysters *Crassostrea virginica* and *A. irradians*, demonstrate a variety of haemocyte responses to ingested toxic algae and associated toxins, including phagocytosis or encapsulation and production of ROS (Hégaret & Wikfors, 2003; Galimany et al., 2008b, c). Hégaret et al. (2011) demonstrated that some toxic algae are cytotoxic to bivalve haemocytes, causing high mortality of haemocytes when exposed *in vitro*; this study concluded that *in vitro* haemocyte bioassays can serve as an efficient screening tool for cytotoxicity of harmful algae to bivalve species.

Despite the ecological importance of ribbed mussels, and the documented impacts of HAB species upon other filter-feeding shellfish, there is a lack of information regarding impacts of HAB species upon the health and filtration activities of *G. demissa*. The lack of studies on HAB interactions with ribbed mussels is probably a consequence of this bivalve not being harvested as a fishery product, but the keystone role this species plays in saltmarsh ecology argues for the need to document possible HAB impacts. The present study was conducted, therefore, to characterize the effects of two HAB species that are found in saltmarsh habitats, *A. anophagefferens* and *H. akashiwo*, upon feeding behaviour, histopathology and haemocytes of *G. demissa*.

**MATERIAL AND METHODS**

**Experimental mussels**

Adult ribbed mussels, *Geukensia demissa* (61–83 mm shell length) were collected at low tide from the Milford Harbor salt marsh, CT, USA. Mussel shells were cleared of epibionts and other encrusting materials. Mussels were held in running, Milford Harbor water containing the natural seston, acclimated from 4 to 16°C at a rate of 2°C per day, and kept at 16°C for 2 weeks. Before the experiment, mussels were fed cultured *Tetraselmis chui* (PLY 429) at a cell density of 10⁶ cell ml⁻¹ for 3 d with a regime of 16 feedings per d (50 ml per feeding), at 90-min intervals.

**Algal cultures**

The HAB species tested in this study were *Aureococcus anophagefferens* (CCMP 1707) and *Heterosigma akashiwo* (OL) obtained from the Milford Microalgal Culture Collection. In addition, the nontoxic alga *T. chui* (PLY 429) was used as a control. The microalgae were cultured in 1.5-l Fernbach flasks. Cultures of *A. anophagefferens* were grown in L-1 medium (Guillard & Hargraves, 1993) and cultures of *H. akashiwo* and *T. chui* were cultured in E medium (Ukeles, 1973). All cultures were maintained at 19 ± 1°C with a photoperiod of 12h:12h light:dark at an intensity of 200 µmols photons m⁻² s⁻¹ PAR. Algal cell densities were determined with an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

In vivo mussel exposure experimental design

One hundred and eighty mussels were distributed haphazardly into 9 6-l glass aquaria, to give 20 individuals per aquarium. Triplicate aquaria of each of three treatments were used: (1) mussels fed cultured *T. chui*; (2) mussels exposed to a culture of *A. anophagefferens* or (3) mussels exposed to a culture of *H. akashiwo*. Each alga was diluted with filtered seawater (FSW) to 5 × 10⁴ cells ml⁻¹ and given with a regime of 16 feedings per d (50 ml per feeding), at 90-min intervals, using computer-automated valves (Smith & Wikfors, 1998). The aquaria were aerated to maintain particle suspension; control experiments without mussels showed that no significant sedimentation of algal cells took place in the aquaria.

Samples of water were collected from each aquarium on day 0 (just after the first exposure to the toxic alga) and after 3 and 5 d of exposure to the experimental microalgal treatments. At each sampling time, CRs were measured in all aquaria. On the last day, 10 mussels from each tank were processed for histopathology and the other 10 mussels were used to determine mean dry tissue weights.

CR measurements

Mussel CR was measured at the same hour each day of sampling using the clearance method according to Coughlan (1969) and reviewed by Riisgård (2001), wherein the filtration activity of the mussels is measured as the volume of water cleared of particles per unit of time. Algal cells were added to each aquarium containing 6 l of FSW (0.5 µM) and 20 mussels. Immediately after the addition of algal culture, the reduction in the number of suspended cells was monitored by taking water samples (5 ml) every 10 min for 90 min and counting the remaining...
suspended particles with an Accuri C6 flow cytometer. After the sample was analysed, the remaining sample water was returned to the experimental aquarium to minimize reduction in the total water volume. To ensure at least a minimum number of algal cells for mussels to feed continuously, the repeated measurements of CR over time were performed by adding algal suspension to restablish the initial algal cell density in each aquarium when depletion of algae occurred; this procedure is similar to that described by Kittner & Riisgård (2005).

The CR was determined from the exponential decrease in algal cell density as a function of time using the formula (Riisgård, 2001):

$$\text{CR} = \left(\frac{V}{n}\right) \ln(C_0/C_t),$$

where $C_0$ and $C_t$ are algal concentrations at time 0 and time $t$, $V$ is the volume of water and $n$ is the number of animals (20 per aquarium). All CR values were standardized to $1\,\text{g}$ of dried mussel flesh using the following equation:

$$\text{CR}_s = \text{CR}_e \times \left(\frac{1}{W_s}\right)^3,$$

where $\text{CR}_e$ is the standardized CR, $\text{CR}_e$ is the experimentally determined CR and $W_s$ is the average dry body mass measured for the mussels. We used a $b$ value of 0.83, as determined by Riisgård (1988) for *G. demissa*.

### Histopathology

On the last experimental day, a 4-mm cross-section of each mussel, including digestive diverticula, gills and mantle, was dissected and fixed in Davidson’s fixative for 24 h at room temperature. The section then was rinsed in 50% ethanol in FSW for 2 h and transferred to 70% ethanol. Samples were dehydrated and embedded in paraffin. After processing, 5-μm sections were stained using a haematoxylin-eosin staining procedure (Howard et al., 2004) and examined under a light microscope.

The mean thickness of the digestive gland tubular epithelium was measured for five mussels from each aquarium (15 mussels per treatment). The measurements involved four readings from each of 10 tubules chosen randomly for each individual using the AxioVision 4.0 image analysis software package (Zeiss).

### In vitro haemocyte exposure

Ribbed mussels acclimated to laboratory conditions as described above, but not exposed to HAB cultures, were selected for *in vitro* haemocyte bioassays. Haemolymph was withdrawn with a 21-gauge needle and a 1-mL syringe from the posterior adductor muscle of each ribbed mussel and stored temporarily in an Eppendorf microcentrifuge tube on ice.

Haemocytes from five ribbed mussels were exposed to the different algal species at a cell density of $10^6$ cells mL$^{-1}$. This concentration was used methodically by Hégaret et al. (2011); repeating this procedure allowed comparisons between the two studies. Control analyses were also carried out on haemocytes in 0.2 μm FSW and the media (L-1 medium and E medium) from the cultures at a quantity equivalent to microalgal culture exposures. All incubations were done at 15°C for 4 h, after which the haemocytes were analysed with a FACSscan flow cytometer (BD Biosciences, San Jose, CA, USA) to evaluate haemocyte mortality, expressed as percentage of dead haemocytes showing propidium iodide fluorescence (Hégaret, Wikfors & Soudant, 2003).

### Statistical analysis

Statistical analyses were performed using the freeware statistical software program R v. 2.13 (http://www.r-project.org), using functions developed by Rand Wilcox (http://www-rusc.edu/~rwilcox/). All tests were performed at the $\alpha = 0.05$ level. A percentile bootstrap method for multiple comparisons of trimmed means was used to evaluate differences across the three treatments, and/or across the three sampling dates, for a variety of variables. The bootstrap method was selected for use instead of the ANOVA $F$-test because the percentile bootstrap method does not assume normality or homoscedasticity, and typically has higher power than the ANOVA $F$-test (Wilcox, 2003). The variables evaluated with the percentile bootstrap method include: (1) mortality of mussel haemocytes after a 4 h exposure to the three algal strains, the two types of media, or FSW; (2) CRs across the three treatments and across the three sampling dates and (3) the thickness of the digestive gland tubular epithelium across algal treatments on day 5. Before analysing the CRs, data were grouped by treatment and sampling date, and outliers were removed within each group using the Hampel identifier, as modified by Rousseeuw & Van Zomeren (1990). CRs were calculated by averaging across repeated measurements of mussels in a single tank on each sampling date. CRs were not able to be calculated from the mussels exposed to *H. akashiwo* on day 5 of the experiment, because sufficient culture of this species was not available. CRs of mussels fed *T. chui* within tank 1 on day 3 were anomalously low; therefore, this tank was excluded from the statistical analysis. The histologically-determined pathologies found were compared on the final day of the experiment, across all three treatments, using the Storer-Kim method for comparing binomials (Storer & Kim, 1990; Wilcox, 2003).

### RESULTS

#### CR measurements

Table 1 shows mean values (+ SE) of standard CRs throughout the experiment. There was a significant increase in clearance of *Tetraselmis chui* as time progressed; the difference between days 0 and 5 was significant ($P < 0.001$), but not between days 0 and 3 ($P = 0.07$) nor between days 3 and 5 ($P = 0.37$). The mussels exposed to *Aureococcus anophagefferens* showed a significant ($P < 0.001$) increase in CR on day 3 but no difference between days 0 and 5. There was no difference between days 0 and 3 for the mussels exposed to *Heterosigma akashiwo*.

Results comparing the different algae on the same day (Table 1) show that mussels feeding on *T. chui* had higher CRs on all three experimental samplings ($P < 0.001$). On day 0, mussels exposed to *A. anophagefferens* had significantly ($P < 0.001$) lower CRs than the mussels exposed to *H. akashiwo*. There was, however, no significant difference ($P = 0.19$) between these two treatments on day 3.

#### Histopathology

Ceroidosis, the accumulation of lipofuchsin pigment (ceroid) that appears yellow to brown in haematoxylin-eosin sections, was a pathological condition observed in all treatments throughout the experiment.

### Table 1. Average of standard CRs ($1\,\text{h}^{-1}\,\text{g}^{-1}$) (+ SE) for each algal diet throughout the experiment.

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tetraselmis chui</em></td>
<td>1.20 ± 0.07$^{a1}$</td>
<td>2.07 ± 0.32$^{a3}$</td>
</tr>
<tr>
<td><em>Aureococcus anophagefferens</em></td>
<td>0.14 ± 0.04$^{a2}$</td>
<td>0.81 ± 0.08$^{b2}$</td>
</tr>
<tr>
<td><em>Heterosigma akashiwo</em></td>
<td>0.63 ± 0.05$^{a3}$</td>
<td>0.60 ± 0.12$^{a2}$</td>
</tr>
</tbody>
</table>

Letters denote statistical differences over time, and numbers indicate differences between treatments.
throughout the entire experiment, with an average prevalence of 32% of individual mussels (Fig. 1). The mantle tissues were observed gametes in stage II of development according to Lubet (1957) in all cases. Nevertheless, most of the mussels exposed to *A. anophagefferens* and *H. akashiwo* showed significant gonad reabsorption after only 5 d of exposure, relative to the *T. chai* control (both *P* < 0.0001), in which gonad reabsorption was not observed in a single mussel (Fig. 1). In mussels challenged with toxic algae, the gametocytes lost structure and were partially reabsorbed (Fig. 2). Mussels exposed to either HAB species showed a significant decrease in the presence of ciliates in the gills, compared with control mussels fed *T. chai* (*P* < 0.001 for *A. anophagefferens* and *P* = 0.013 for *H. akashiwo*) (Fig. 1). Measurements of digestive tubule thickness (Fig. 3) showed differences between the mussels exposed to the benign and both harmful algae (*P* < 0.001), with average values for mussels fed on *T. chai*, *A. anophagefferens* and *H. akashiwo* (± SE) of 23.81 ± 0.26, 19.72 ± 0.20 and 19.02 ± 0.20, respectively.

In vitro haemocyte bioassays
Haemocytes exposed to the experimental treatments showed differences in mortality (Fig. 4). The lowest mortality was observed for the haemocytes exposed to the two different culture media (L-1 medium and E medium), showing no significant differences between them. These two values were significantly (*P* = 0.02) lower than that of haemocytes exposed to FSW, but the mortality in FSW was still very low, only 7%. Haemocytes exposed to *T. chai* had significantly (*P* < 0.001) higher mortality, with a mean of 20%. Nevertheless, this value was significantly (*P* < 0.001) lower than the mortality found for the haemocytes exposed to *A. anophagefferens* or *H. akashiwo*, with mean values of 49 and 64%, respectively. There was no significant difference in mortality of haemocytes exposed *in vitro* to either HAB species (*P* = 0.0645).

**DISCUSSION**
This study showed that the feeding behaviour and haemocytes of ribbed mussels are negatively affected when mussels are exposed to the toxic alga *Aureococcus anophagefferens* or *Heterosigma akashiwo*. Ribbed mussels decreased their CR when feeding on each HAB species. To our knowledge there are no previous studies measuring the CRs of *Gekkensia demissa* when feeding on toxic algae. Early reports studying the feeding behaviour of northern quahogs *Mercenaria mercenaria* and blue mussels *Mytilus edulis* exposed to *A. anophagefferens* indicate reduced feeding for both bivalves (Tracey, 1988; Bricelj, MacQuarrie & Schallifer, 2001). There is evidence that the inhibition of clearance in bivalves exposed to *A. anophagefferens* is caused by the release of a dopamine-mimetic compound from the extracellular mucus surrounding *A. anophagefferens* cells, which interferes with the function of the lateral gill cilia (Gainey & Shumway, 1991). Nevertheless, *A. anophagefferens* was shown to have no effect on the activity of the lateral gill cilia of *G. demissa* (Gainey & Shumway, 1991).

The small size (2 μm) of *A. anophagefferens* cells may have contributed to the decreased CR observed in our study, as the retention efficiency of cells of this size is reduced to 70% (Risgård, 1980). If this is true, then the higher clearance of *A. anophagefferens* on day 3, compared with days 0 and 5, could have resulted from aggregation of the *A. anophagefferens* culture (Maloney et al., 2003) used on day 3 resulting in larger effective particle size, although this cannot be confirmed because no observations of cell clumping were recorded. Nevertheless, the CRs found for *G. demissa* when exposed to *A. anophagefferens* are much lower that the control, suggesting that some other factors might contribute to the feeding behaviour observed. The cell surface of the Texas brown tide species, *Aureoumbra lagunensis*, has a layer of exopolimer secretion and high levels of this substance have been shown to reduce feeding in some protozoa. It is speculated that a
Previous reports of bivalve exposure to *H. akashiwo* showed species-specific responses; oysters (*Crassostrea virginica*) remained closed, but *M. edulis* and *M. mercenaria* were open and producing biodeposits (Hégaret, Shumway & Wikfors, 2007). The ichthyotoxicity of this species, and other raphidophytes, has been attributed to oxidation of algal eicosapentaenoic acid by ROS generated by algal cells disturbed by the turbulence of passing through the gills (Marshall, Nichols & Hallegraeff, 2002). A reduction in retaining this species and, consequently, reducing the CR, would decrease exposure of mussel gills to this toxic effect, thereby diminishing the impact of this alga on the mussels. Cytotoxicity of *H. akashiwo* to bivalve haemocytes in vitro has been demonstrated previously (Hégaret et al., 2011) and confirmed in the present study for ribbed mussels, reinforcing the advantage to the mussels of minimizing contact with the cytotoxic cells by reducing filtration.

Previously reported bivalve CRs of nontoxic algae are diverse. Some studies have reported similar values to our observations for *T. chui* (Wright et al., 1982; Lonsdale et al., 2009). Other authors have reported higher CRs for similar-sized mussels (Jordan & Valiela, 1982; Kemp, Newell & Krambeck, 1990). The different CRs reported in other studies are likely attributable to different nutritional value of the food in the diet provided (Pales Espinosa, Allam & Ford, 2008). The increase in CR of *T. chui* over time in the present study may be related to habituation to the periodic feeding regime over time; we have observed this before in nutritional studies with several bivalve species (Wikfors et al., 1996).

We observed that mussels exposed to both toxic algae showed thinner digestive tubules than the mussels ingesting *T. chui*. Thinner digestive tubules are a sign of starvation and have been reported previously in bivalves exposed to toxic algae (Brícelj, MacQuarrie & Smolowitz, 2004; Galimany et al., 2008a). Therefore, we hypothesize that thinner digestive tubules may be attributable, entirely or in part, to starvation during the 5-d exposure to HABs.

Bivalves have been shown to hasten the defecation of toxic or noxious phytoplankton by postigestive selection (Hégaret, Shumway & Wikfors, 2007). Histopathology also showed gonadal reabsorption in mussels exposed to *A. anophagefferens* or *H. akashiwo*. Tracey (1988) observed reproductive failure in *M. edulis* after a brown tide in Narragansett Bay (USA). As gametogenesis is an energy-demanding process, the reabsorption of gametes would redirect energy from gametogenesis to defense mechanisms against stressful conditions, i.e. the exposure to toxic algae (Bayne et al., 1978; Galimany et al., 2008c). Gill ciliates live between the gills of mussels and establish a commensal relationship causing no harmful effects on the bivalves (Bower, 2007). In our study there was a decrease in occurrence of gill ciliates in the mussels exposed to *A. anophagefferens* or *H. akashiwo*. Similar findings were reported by Hégaret et al. (2009) when Manila clams with *Perkinsus olseni* infection were exposed to the toxic alga *Prorocentrum minimum*. It is possible that the gill ciliates were impacted directly by the HAB species; *H. akashiwo* has been shown to be toxic to free-living ciliates in laboratory culture (Clough & Strom, 2005) and *A. anophagefferens* can produce substances that inhibit or retard prostanoid grazing activities during the period of bloom initiation (Caron et al., 2004).

Ceroidosis occurs in many animal phyla, including molluscs, and is associated with disturbances in lipid metabolism in several pathological conditions caused by nutritional deficiencies, toxicity or disease (Wood & Yasutake, 1956). Thus, the background level of ceroidosis found in all of the mussels (even the *T. chui* controls) could have been caused by many factors and was unlikely to have been related to the presence of the HAB species.

*In vitro* haemocyte bioassays are a useful screening method for possible harmful physiological effects of HABs on bivalves.

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**Figure 3.** Optical micrographs of digestive gland tissues sampled on day 5, from *Geukensia demissa* exposed to *Tetraselmis chui* (A) and *Heterosigma akashiwo* (B). dg, digestive gland. Arrows point to digestive gland epithelium.

**Figure 4.** Mean value of the mortality percentage of haemocytes of *Geukensia demissa* (± SE) incubated with the following triplicate treatments: L-1 medium (Med 1), E medium (Med 2), filtered sea water (FSW), *Tetraselmis chui* (T. c), *Aureococcus anophagefferens* (A. a) and *Heterosigma akashiwo* (H. a). Letters denote statistical differences between treatments.
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